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14. ABSTRACT

Pain is a common and distressing symptom that impacts the quality of life of many patients with neurofibromatosis. The pain is often due to the formation of a neuroma. To understand better how neuromas cause pain and what treatments may be provided, we have developed an animal model of a painful neuroma. The tibial neuroma transposition (TNT) model has been confirmed as a model of neuropathic pain. The TNT model has been established as reliable and valid (Specific Aim 1). In the TNT model, the neuroma test-site mechanosensitivity is dependent on neural input from the tibial neuroma. In the TNT model, hindpaw mechanical hyperalgesia is independent of input from the tibial neuroma. We have altered the formation of a neuroma by applying a toxin that is retrogradely transported (suicide transport) leading to neuronal death and axonal death (Specific Aim 2). Application of target specific toxins will lead to death of various populations of axons. The TNT model clearly demonstrates that axons surviving suicide transport can maintain the pain behaviour associated with neuroma sensitivity. Complete obliteration of axons in the robust TNT model is required for cessation of neuroma pain (Specific Aim 3)

15. SUBJECT TERMS
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INTRODUCTION

Pain is a common and distressing symptom that impacts the quality of life of many patients with neurofibromatosis. The pain is often due to the formation of a neuroma. To understand better how neuromas cause pain and what treatments may be provided, we have developed an animal model of a painful neuroma. The tibial neuroma -transposition (TNT) model has been confirmed as a model of neuropathic pain. The TNT model has been established as reliable and valid (Specific Aim 1). In the TNT model, the neuroma test-site mechanosensitivity is dependent on neural input from the tibial neuroma. In the TNT model, hindpaw mechanical hyperalgesia is independent of input from the tibial neuroma. We have altered the formation of a neuroma by applying a toxin that is retrogradely transported (suicide transport) leading to neuronal death and axonal death (Specific Aim 2). This technique has been refined using different target-specific toxins, varying the delivery method and examining subsequent pain behaviour (Specific aim 3).

BODY

We will present a summary of our efforts that represent 1) research based directly on the 3 specific aims of the grant and 2) outgrowth research to improve methodology in this work and increase our understanding of the patho-physiology underlying neuropathic pain.

1) Specific Aim Directed Research

In year one, we firmly established the TNT model with the addition of sufficient animal numbers to our preliminary work to produce a reliable, statistically significant result. We then completed our first specific aim by demonstrating that blocking neural input from the neuroma to the CNS reversed the pain behavior produced by the TNT model. In year 2 we experimented with a variety of neural toxins to prevent neuroma formation through retrograde transport and cell death. In year 3 we continued experimenting with a variety of neural toxins and altered delivery methods in an attempt to achieve suicide transport and reverse pain behavior.

1) **Specific aim #1: Does blocking neural input from the neuroma to the CNS reverse the pain behaviors produced by the TNT model?** As indicated in the year one progress report, this specific aim has been completed. Injecting an anesthetic at the site of the neuroma or cutting the nerve proximal to the neuroma reversed the neuroma tenderness produced by applying mechanical stimuli at the neuroma site. However, these manipulations did not reverse the mechanical hyperalgesia on the paw.

2) **Specific aim #2: Develop a technique to selectively prevent neuroma formation with OX7-saporin.** As indicated in the year two progress report, we did not obtain a reproducible decrease in behavioral signs of pain when OX7-saporin was injected into the nerve. During the second and third year, we explored two different strategies to overcome this difficulty. The first strategy was to use different neural toxins. The second strategy was to employ different techniques for administering the neural toxins.

3) **Specific aim #3: Does OX7-saporin prevent or reverse the pain behaviors produced by the TNT model?** During the numerous experiments performed during the last two years, we have evaluated both neuroma formation (histology) and neuroma pain (behavior) when doing each of our experiments. We have found that if the neural toxin does not result in complete prevention of axonal sprouting and neuroma formation, the pain behavior persists. We also tried combinations of various toxins to target different populations of axons.

A brief summary of the results of these experiments is provided below.

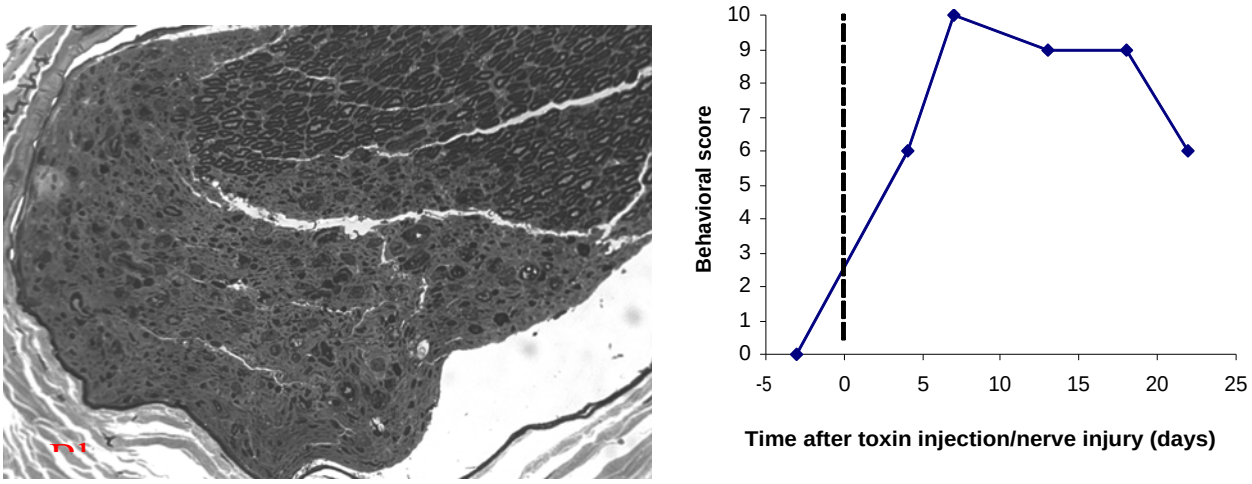
a. Use of different neural toxins. After discussion with various experts in the field of neural toxins and retrograde transport, we identified two other neural toxins that may be effective: Wheat Germ Agglutinin

(WGA) coupled to saporin and cholera toxin B (CTB) coupled to saporin. WGA binds to unmyelinated fibers and should lead to selective loss of unmyelinated fibers. CTB binds to large myelinated fibers and should lead to a selective loss of myelinated fibers.

WGA-saporin was injected into the tibial nerve (in doses ranging from 5 to 200 ng in 2 ul), the nerve was ligated distal to the injection and rotated to the lateral position (using our standard approach for producing the TNT model). Behavioral testing for 1 to 3 weeks showed variable results. Most animals, showed little evidence for an analgesic effect of the injection. Even at the higher doses, some animals showed modest anaglesia and others none at all. Despite the lack of reproducible behavioral effects, the histological samples from the proximal nerve showed evidence for degeneration following the neural toxin. Similar results were obtained when CTB-saporin (in doses ranging from 0.03 to3.0 ug in 2 ul) was injected into the tibial nerve.

The pain behavior from neuroma formation did not reverse when either CTB or WGA toxin was employed. It was felt that preservation of either the mylinated or unmyelintaed could be signaling the pain and thus we decided to move ahead with a new series of experiments. We tried a mixture of CTB and WGA at various doses in an attempt to prevent regeneration of both classes of fibers. This still did not consistently reverse the pain behavior.

A "breakthrough" in our thinking on this came when we investigated histological samples taken close to the neuroma site (i.e., 3 mm proximal to the ligature). The figure below shows the results in one animal following injection of 3 ug of CTB-saporin into the tibial nerve. As shown

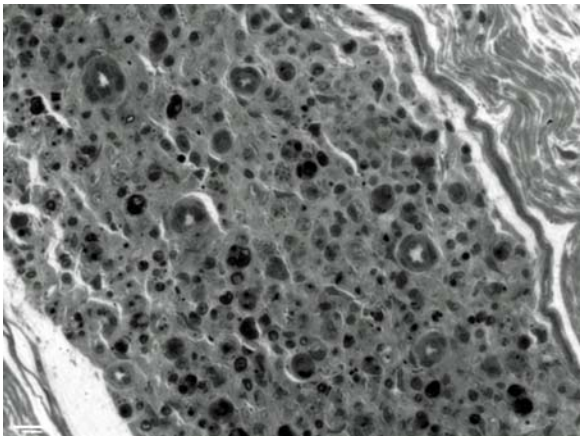


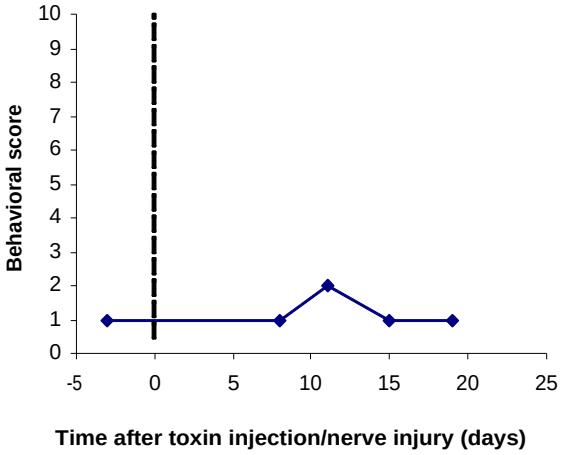
in the left panel, pronounced degeneration is seen in part of the nerve, but the other part of the nerve is relatively spared. This suggests that the micro-injection of the neural toxin was restricted to one fascile in the nerve and the toxin did not cross over to adjacent fasicles. In the right panel, the behavioral response following mechanical stimulation at the neuroma site is plotted as a function of time after nerve injury (and toxin injection). This animal reach the maximum behavioral score (i.e., 10) and stayed at a high behavioral score throughout the three week testing period. Thus, no obvious signs of analgesia were apparent. Our interpretation of these results is that the spared fascile innervated the neuroma and provided sufficient neural signaling to produce the behavioral response. Base on this observation, we set out to develop alternate techniques for administering the neural toxins that would lead to a complete denervation of the tibial nerve and reversal of pain behavior (see next section).

b. <u>Development of different techniques for administering the neurotoxins.</u>

It appeared that the neural toxin was respecting the perineurial barrier. The first idea to overcome this problem was to crush the nerve prior to injecting the neural toxin. We reasoned that the crush would disrupt the perineurium and provide access of the neural toxin to all of the fasicles. Unfortunately, this technique did not result in improved behavioral responses.

The next idea was to micro-inject each of the fasicles in the tibial nerve. An example of the outcome of this experiment in one animal is shown below.





In this case, there was complete degeneration of the tibial nerve (left panel) and only a weak behavioral response (right panel). However, this procedure proved to be technically very challenging since it was difficult to insert the needle into some of the smaller fasicles. In addition, we still had animals that had incomplete degeneration and showed no behavioral signs of analgesia.

Our next idea was to place the cut nerve into a pool (or "well") of neurotoxin solution. This would expose all fasicles to the neural toxin. To achieve this, the tibial nerve is cut and ligated. The suture is used to pull the nerve thru a PE50 tubing. The nerve and tubing is then cut proximal to the ligature and the nerve is pulled back into the tubing so that a 1.5 mm empty tubing space is formed that serves as a drug loading pool just distal to the nerve stump. The distal end of the tubing is closed with a tight ligature. A glass micropipet is used to load this space with the toxin. The nerve is exposed to the toxin for 1 - 2 hours. Then, the tubing is removed, and the nerve is ligated and rotated to the lateral position (as per our normal TNT procedure). Our initial results with this technique were promising. A series of experiments were performed using a dose response escalation. Unfortunately we were unable to prevent complete axon regeneration and neuroma formation. In addition, the pain behavior persisted.

Our next idea was to produce a more permanent and physiologic pool for the drug delivery. We developed a surgical technique utilizing the anatomy and physiology of the peripheral nerve. In the TNT model, the nerve is divided and a neuroma forms at the cut end. We modified this procedure in the following manner: there is a natural pressure gradient across the perineurium due to tight junctions in the wall similar to the blood brain barrier. When a nerve is cut, the contents of the fascicles will begin to emanate out the end. When we cut the end of the tibial nerve, we allowed the contents of the fascicle to pouch out and at the same time we retracted the epineurium. The extruded neural material was removed and then the epineurium was brought forward and closed creating a potential space. A micropipette was used to fill the space with drug forming a repository. This provided the drug excellent exposure to the cut end of the axons.

A series of experiments using this epineurial well technique were performed. We also wanted to know if gently crushing the end of the nerve prior to administering the drug would have an effect on the ability of the drug to enter all targeted axons. For example, in one experiment, the repository was filled with CTB-saporin + WGA-saporin (0.3 ug/ul + 100ng/ul). Twelve animals were divided into four equal groups: n=3 with PBS, n=3 nerve with non-crushed CTB-SAP + WGA-SAP; n= 3 CTB-SAP + WGA-SAP pooling without injection; n=3 nerve crushed additional micro-injection with CTB-SAP + WGA-SAP into exposed fascicles. The results were

consistent in that behavior correlated with histology. The pain behavior would only reverse in a few animals. These animals had the most robust histo-pathology with loss of axons and little or no regenerating axons. Unfortunately, we have not been able to modify the delivery technique sufficiently to result in a consistent loss of axonal innervation to the neuroma. To ensure that the animals do reverse behavior when the neuroma is dennervated, a final series of experiments were performed using the epineurial well technique but adding a control arm in which the animals underwent proximal cut of the tibial nerve at various time points. Each of the control animals demonstrated loss of neuroma sensitivity with tibial nerve cut but preservation of hind paw mechanosensitivity. The experimental animals again demonstrated variable histo-pathology and behavior that tended to correlate with the degree of axonal degeneration and lack of regeneration.

2) Outgrowth Research

Several pharmacologic preparations that inhibit neuronal activity have been developed to treat epilepsy. Many of these preparations have also proven to have utility in the treatment of neuropathic pain.

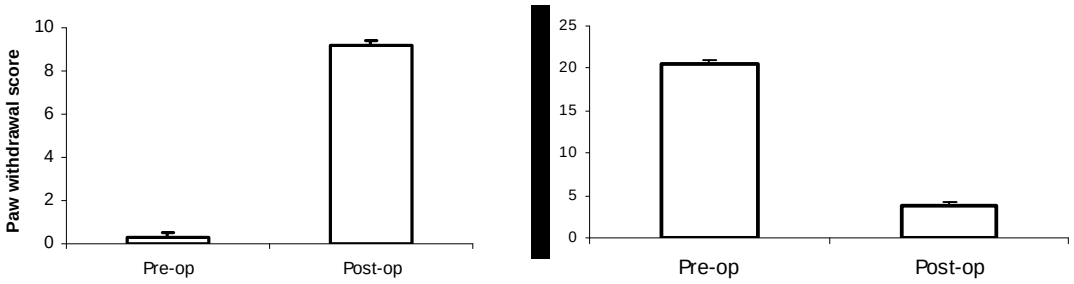
a) Pregabalin

Pregabalin (trade name Lyrica) is an alpha$_2$-delta (α_2-δ) ligand that has analgesic, anticonvulsant, and anxiolytic activity. The drug is widely used in the clinical treatment of neuropathic pain and pain from neuroma formation. Systemic administration of lidocaine has also been used to treat neuropathic pain. We performed an experiment to compare the effect of pregabalin(PGB), morphine, and lidocaine(LDC) on the TNT model.

Method: TNT model surgery was performed on 48 rats. Systemic doses of PGB (2-40mg/kg IP), morphine (0.5 – 8mg/kg IP), and LDC (2-40mg/kg IP) were administered. The experiments were conducted in a blinded, randomized fashion. On a given test day, each rat received an intraperitoneal injection of one dose of either pregabalin, lidocaine, morphine or saline vehicle. Behavioral testing for neuroma test-site mechanosensitivity and hindpaw mechanical hyperalgesia was performed immediately before drug administration and at five time points after the injection. Each animal was tested with one dose of each drug (with a least a two day wash out period).

Results: 96% of the TNT created animals developed the neuroma test site mechanosensitivity and the hindpaw mechanical hyperalgesia to innocuous mechanical von Frey hair stimulation of neuroma and the lateral side of the hindpaw ipsilateral to the formed neuroma. The average paw withdrawal threshold (g) was decreased from 20.5±1.4 to 3.7± 0.4, and the average paw withdrawal score was increased from 0.28± 0.15 to 9.1 ± 0.25 P<0.001(Fig. 1). The animals that did not display the pain behavior were excluded from the study

Fig. 1 A Fig. 1 B



Effect of morphine: At the highest dose of morphine (8 mg/kg, I.P.) administered, the animals were not sedated. Systemic administration of low doses morphine (0.5, 1, 2 mg/kg, I.P.) had no effect on paw withdrawal up to 240 min post-injection (compared to vehicle). In contrast, injection of morphine at 8 mg/kg significantly decreased the hindpaw withdrawal score 30 min after injection 0.27 ± 0.12 ($P < 0.01$, compared to vehicle), and this effect continued up to 180min. The second highest dose of morphine also had a significant effect on blocking neuroma pain starting from 30 min post-injection and lasting to 90 min 0.39 ± 0.14($P < 0.05$). The

effect of morphine on the neuroma test-site were dose-dependent, and more effective than the effects of PGB and LDC (Fig. 2).

Fig 2. A

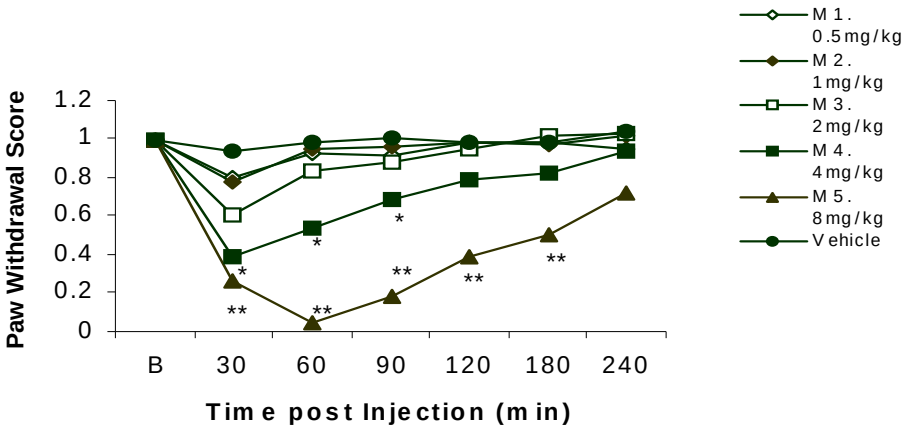
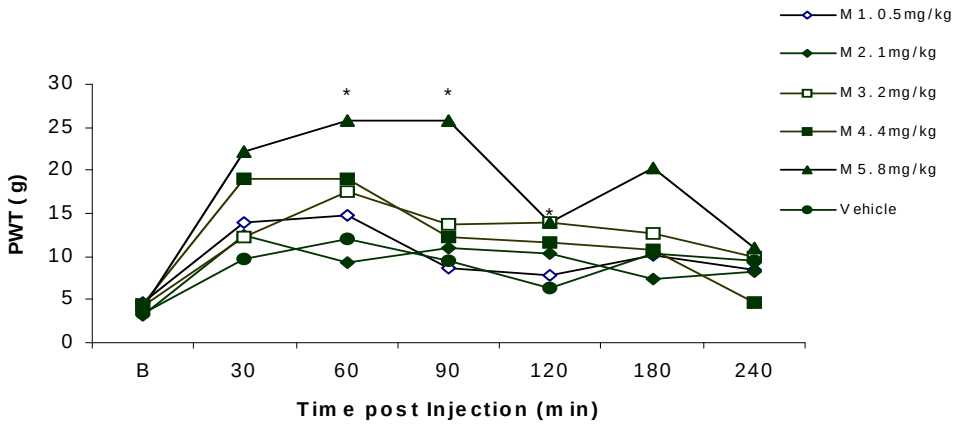


Fig 2 B



Effect of Pregabalin: Systemic injection of PGB (2 - 40 mg/kg, IP) to TNT rats did not result in any animal change in posture. No animal developed incontinence or somnolence. The highest doses of PGB (40 mg/kg) had a significant effect on attenuating the neuroma test-site mechanosensitivity 30 min (0.55 ± 0.12 ($P < 0.05$)) after injection, and this effect continued up to 60 min (compared to vehicle). From 60 min up to 120 min after administration of the highest dose, the hindpaw mechanical hyperalgesia was attenuated compared to baseline (15.73 ± 4.78 ($P < 0.05$)). No significant effects was observed in the 240 min testing period after application of PGB in the lower doses (2, 4, 10, 20mg/kg I.P.) on either testing site (Fig 3. A. B)

Fig 3. A

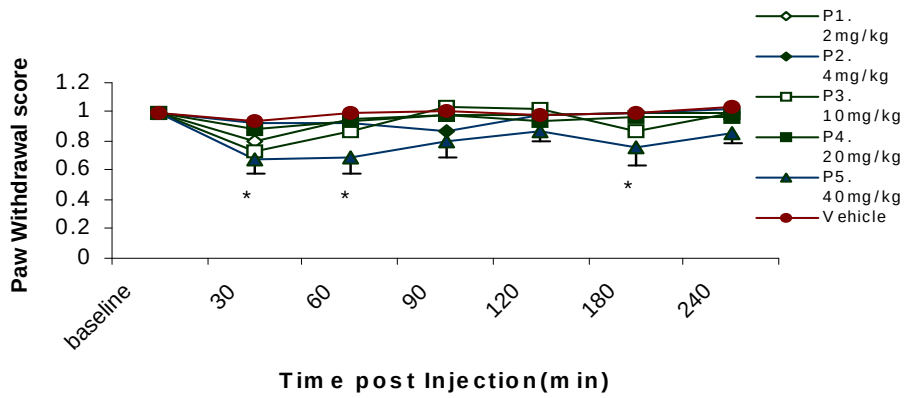
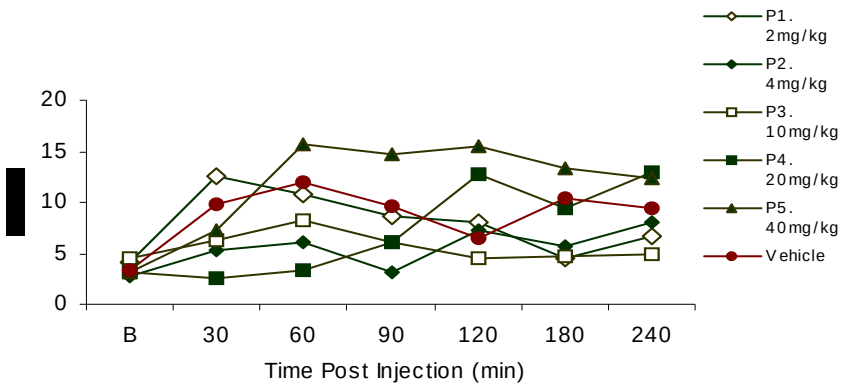


Fig3. B



Effect of Lidocain: Animals given a systemic injection of LDC (40 mg/kg, I.P.) showed no signs of sedation or incontinence. Administration of lower doses LDC (2, 4, 10, 20mg/kg, IP) had no effect on the neuroma test-site mechanosensitivity and the hindpaw mechanical hyperalgesia up to 240 min post-injection compared with either the baseline response or injection of vehicle. In contrast, 30 min post-injection of the highest dose LDC (40 mg/kg) significantly decreased the paw withdrawal score on neuroma testing (0.55±0.12 ($P < 0.05$)) compared with vehicle. In contrast, the same dose of LDC (40 mg/kg) had no effect on the hindpaw mechanical hyperalgesia (Fig. 4 A. B).

Fig. 4. A

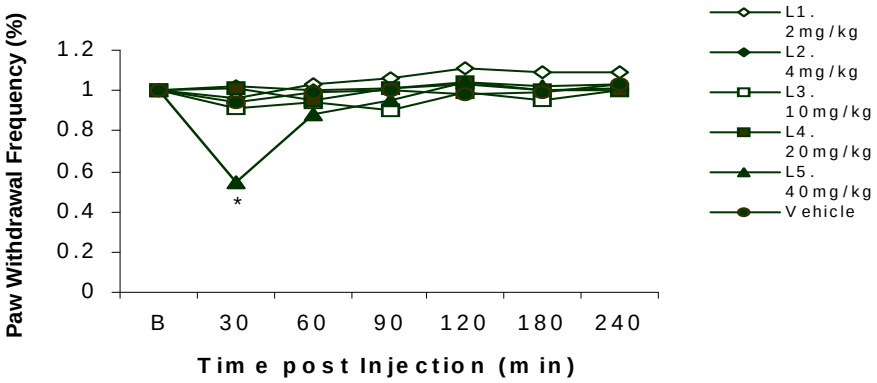
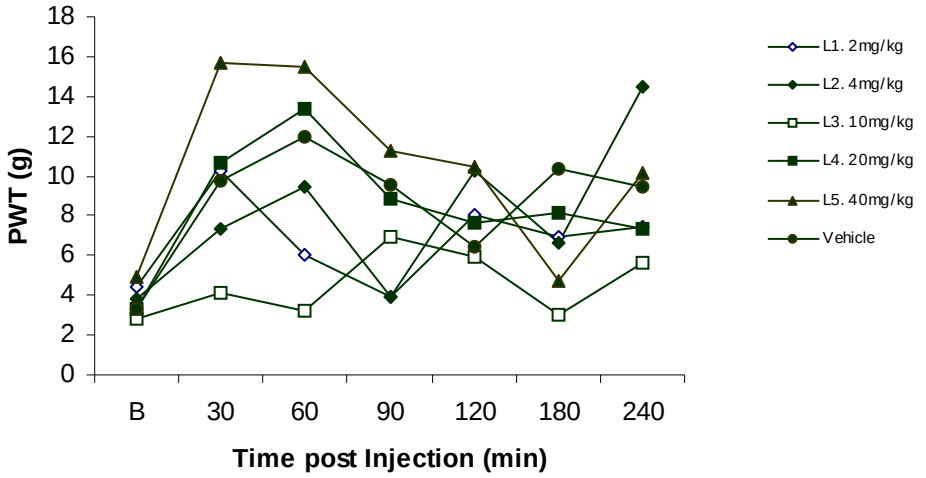


Fig. 4. B



Conclusion: This study demonstrated that pregabalin, lidocaine, and morphine all have an effect on pain behavior produced by the TNT model. Morphine at the highest dose, decreased the mechanical hyperalgesia in the hind paw. Pregabalin and lidocaine, at all doses tested, had no significant effect on the hindpaw mechanical hyperalgesia (fig 5). The hindpaw hyperalgesia is thought to represent activity in uninjured afferents which overlap territory with injured afferents that have undergone Wallerian degeneration. Morphine administration resulted in a dose-dependent decrease in neuroma sensitivity, while pregabalin and lidocaine's effect were seen only at maximal dose. Neuroma test-site mechanosesitivity is thought to represent mechanical sensitivity of injured afferents and thus should respond to PGB and LDC treatment. This experiment, utilizing the TNT model, has very nicely separated out two forms of pain behavior, each with a distinct pathophysiology and response to treatment. This data is being readied for publication.

Fig 5.A

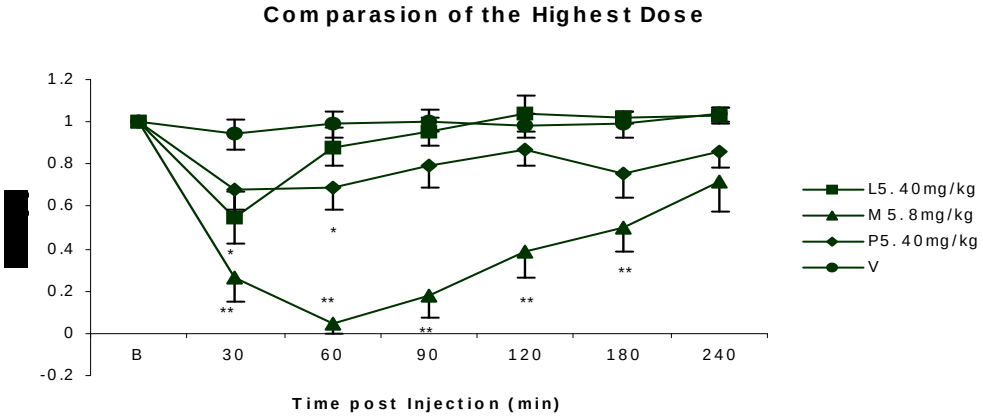
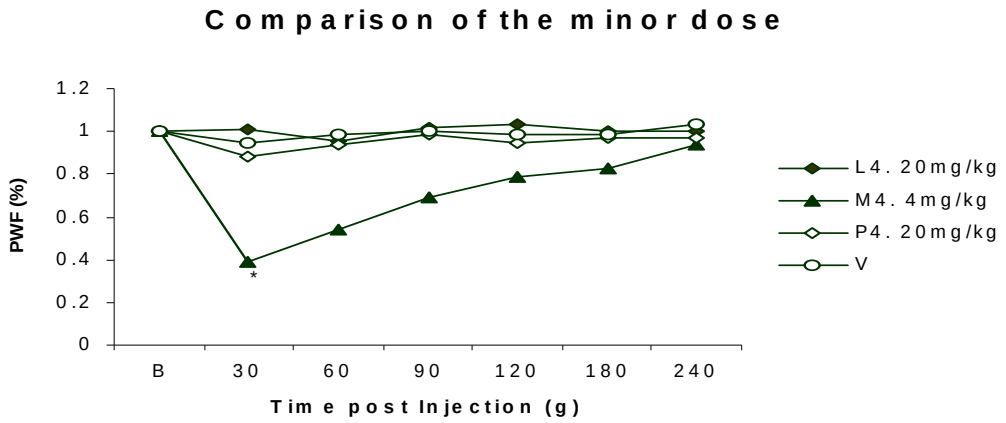


Fig. 5 B.

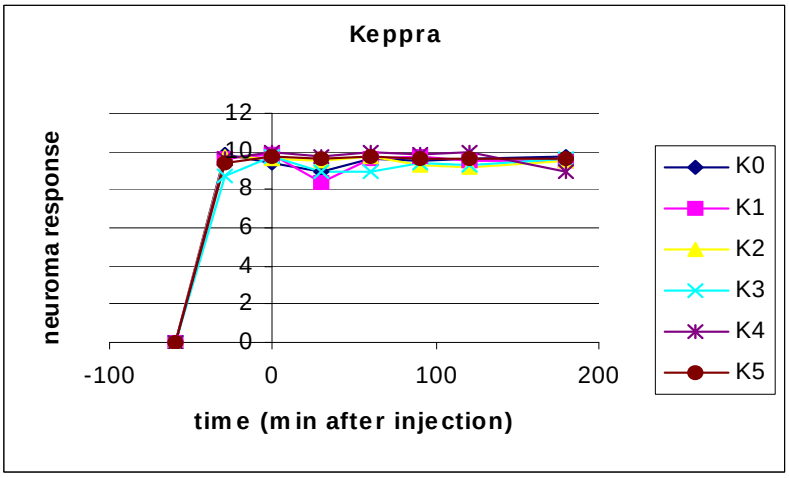


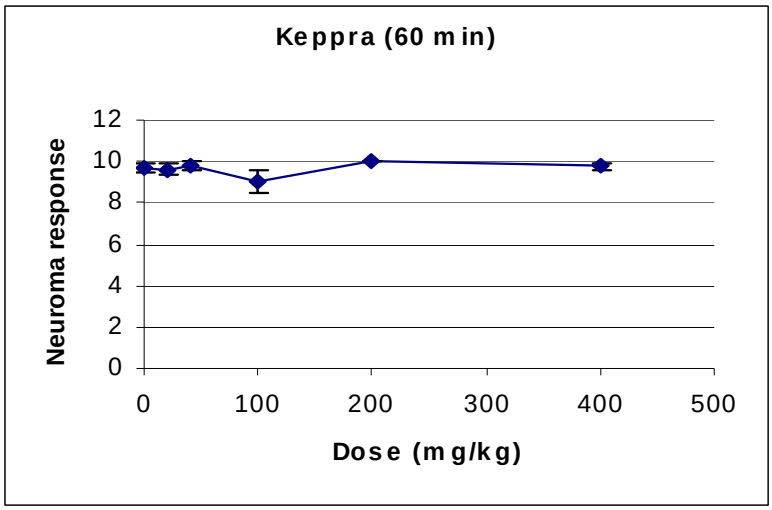
b) UCB Pharmacy.

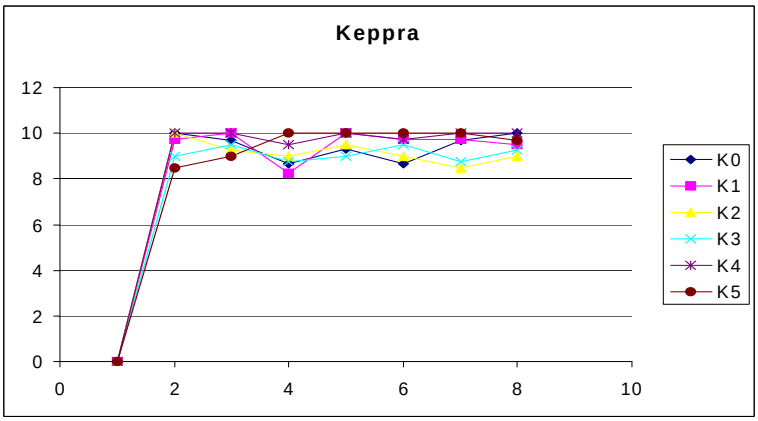
Purpose: Levetiracetam (trade name Keppra) is used to treat partial onset seizure disorder. The manufacturer, UCB Pharama, has been exploring other indications including possible treatment of neuropathic pain. The TNT model was used to test the analgesic effect of Keppra. UCB Pharma supplied the drug.

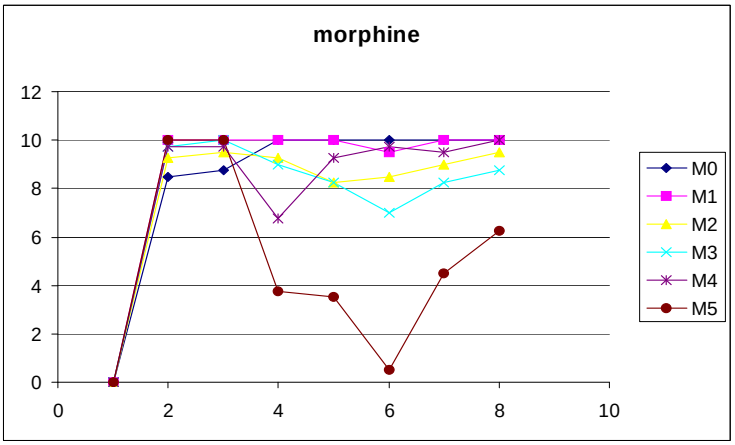
Method: Animals received a systemic administration levetiracetam (20, 40, 100, 200, 400 mg/kg IP), morphine (0.5, 1, 2, 4, 8 mg/kg), or vehicle in a blinded, random fashion. Behavioral testing was performed before surgery, day 6 postoperative, and then on drug delivery days 9 and 15 postoperative. A given animal was tested with one dose of each drug (with at least a two day wash out period). Each dose was tested on eight animals.

Results: Mechanical hyperalgesia in the hindpaw and neuroma sensitivity was not affected by the systemic administration of vehicle or levetiracetam. In contrast, administration of morphine led to a dose-dependent decrease in the frequency of paw withdrawal to mechanical stimulation of the neuroma and increase in paw withdrawal threshold to stimulation of the paw.









Note; for X axis, 1 = pre surgery, 2 = post surgery, 3 = before treatment, 4 = 30 min post, 5 = 60 min post, 6 = 90 min, 7 = 120 min, 8 = 180 min

Conclusion: These results indicate that levetiracetam, in contrast to morphine, does not induce an antihyperalgesic effect in the TNT model of neuroma pain.

c) Collaboration 1

We are collaborating with Michel Kliot MD, Professor Dept. of Neurosurgery, University of Washington. He is the chief of neurosurgery at Puget Sound VA Health Care Center. He and his co-investigators have obtained a grant from the Veterans Administration.

The following is a relevant portion from their proposal.
Our long-term goal is to change the paradigm of how patients presenting with pain due to focal damage to peripheral tissues are diagnosed. We have developed a new focused ultrasound (FUS) based technology that we call transcutaneous acoustic palpation (TAP) that promises to be far more specific than physical examination and diagnostic imaging in identifying pain generators that are deep within the body. We have already demonstrated in two animal models generating superficial sources of pain that FUS can reliably distinguish the tender from the non-tender extremity. We have also demonstrated that we can apply FUS under ultrasound-image guidance. **As a next logical step, we propose to demonstrate that FUS can identify a deeper source of focal pain using the subcutaneous *tibial neuroma transposition model* developed by Belzberg and colleagues (Dorsi et al 2007).**

This work is now being performed. It is possible that this technique of localizing a deep pain generator can be applied to differentiating which tumor in a patient with NF1 is the one causing pain.

d) Collaboration 2

We are collaborating with Dr. Strauch from Columbia University Medical Center on a novel method of neuroma formation. His group is applying cyanoacrylate, a glue-like compound, as a nerve cap to halt the normal progression of neuroma formation. In a preliminary study utilizing the TNT model, the cut end of the tibial nerve was treated with either bipolar coagulation, untreated, or application of the cap. There did appear to be a difference in the neuroma formation and behavior response in the experimental group. Further studies are now underway. The preliminary data was recently presented at the ASPN 2010 annual meeting.

KEY RESEARCH ACCOMPLISHMENTS

- The TNT model of neuropathic pain has been established and is now being used by various research groups to explore treatment options for neuropathic pain.

- The formation of a neuroma subsequent to axotomy can be altered by using retrograde transport of a neural toxin in the proximal stump.

- Neuroma test-site mechanosensitivity can be altered by retrograde transport of a neural toxin.

- The pain behavior associated with neuroma formation may not be dependent on ongoing activity in small fiber neurons (C-fibers, A-delta fibers).

- Reversal of pain behavior associated with neuroma formation may require the ablation of all innervating axons regardless fiber size or class.

- Neuropathic pain that is secondary to neuroma formation is responsive to morphine.

- Neuropathic pain that is secondary to intact fibers overlapping areas of axonal injury and Wallerian degeneration is responsive to morphine.

REPORTABLE OUTCOMES

Personel

This grant has in part supported: Allan J Belzberg, Richard Meyer, Lun Chen, Beth Murinson
Belzberg is a neurosurgeon at Hopkins. He has continued to actively treat and research patients with pain due to neuroma and patients with NF.
Belzberg is collaborating on research with Drs. Kliot and Strauch. Preliminary data from this grant has been used in subsequent grant applications for these collaborations.
Meyer has recently retired.
Dorsi, a Hopkins neurosurgery resident, is performing fully funded research at UCLA looking at neuropathic pain due to peripheral nerve injury. This grant provided preliminary data.
Murinson is a fully funded neurologist at Hopkins looking at various mechanisms of neuropathic pain. This grant provided preliminary data
Chen has completed his research position and has been training to be a physician assistant.

Research Funding

Partial funding from UCB pharma was obtained to perform levetiracetam experiments.

A grant from the Veterans' Administration has been obtained to continue the collaboration with Dr. Kiot from the University of Washington

A grant has been submitted to further explore novel techniques of neuroma formation including use of various "glue caps" in conjunction with Columbia University

Presentation of research material

Dr. Belzberg was the invited guest speaker at the America Society of Peripheral Nerve annual meeting held in Hawaii, January 2009. He provided the *presidential guest lecture* entitled
Neuropathic Pain: from bench to bedside and back again
The work of this grant was heavily featured in the talk and the DOD grant / support acknowledged. The TNT model and results of various treatment interventions was used to explain how neuroma formation can lead to neuropathic pain.

Publications

Abstracts

Dorsi M, Belzberg AJ, Meyer R, Chen L
Management of the Painful Nerve
Spine & Peripheral Nerve Section Meeting, Phoenix, 2007

Chen L, Meyer R, Dorsi M, Belzberg AJ
The TNT neuroma model
NF Tumor Foundation Meeting, Florida, 2008

Chen L, Meyer R, Dorsi M, Belzberg AJ
Effect of Levetiracetam and Morphine in an Animal Model of Neuropathic Pain
ASPN Annual Meeting, Puerto Rico, 2008

Shin, Akelina, Yao, Cadreanu, Strauch
Novel intraoperative application of cyanoacrylate for the prevention of painful neuroma formation
ASPN annual meeting, Florida, 2010

Peer Review

The tibial neuroma transposition (TNT) model of neuroma pain and hyperalgesia.
M. Dorsi, L. Chen, B. Murinson, E. Pogatzki-Zahn, R. Meyer, A. Belzberg

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Peer Review - in preparation

Effects of systemic pregabalin and lidocaine on neuroma sensitivity and mechanosensitivity

CONCLUSION

The tibial neuroma transposition (TNT) model provides the scientific community an animal model of neuroma pain. The pain behavior displayed by the animal results from mechanical stimulation of the neuroma, a phenomenon commonly seen in patients with painful neuroma. The model also provides the ability to study pain related to stimulus evoked behavior in an area of partial dennervation. The TNT model is the only animal model that separates out these two pain phenomenon and allows them to be individually manipulated. This model has now been used by other laboratories to study pain due to nerve injury and neuroma formation.

The application of Ricin to the nerve will result in retrograde transport of the neural toxin and axonal degeneration, a phenomenon that has been coined "suicide transport". There is a dose dependent loss of axons and prevention of neuroma formation. When there is complete obliteration of axons and prevention of any regenerating axons, there is the prevention of a painful nueroma.

The application of Wheat Germ Agglutinin coupled to saporin to a nerve will result in retrograde transport of the neural toxin and loss of small fiber axons. The loss of these “pain fibers” did not result in a loss of pain behavior. The application of cholera toxin B coupled to saporin to a nerve will result in retrograde transport of the neural toxin and loss of large fiber axons. The loss of these fibers did not result in loss of pain behavior. Combining these two target specific toxins will result in a loss of targeted nerve. Despite a loss of pain related fibers, the neuroma that forms is still associated with pain behavior. It would appear that a neuroma can result in pain behavior despite having what are thought of as "the pain fibers" removed from the neuroma. The use of suicide transport to prevent painful neuroma formation awaits the development of better toxins that target axons and are safe.

The treatment of patients suffering from neuropathic pain remains a challenge. There is resistance to using opiates (such as morphine) in these patients. The experiments performed in this research project have demonstrated that in an animal model of neuropathic pain (the TNT model), the use of system morphine does decrease pain behavior emanating from the neuroma and from the region of mechanical hyperalgesia (partially dennervated skin). Further, systemic lidocaine can be expected to impact neuroma sensitivity related pain but not the mechanical hyperalgesia. This research project can help support the use of various pharmacologic agents in neuropathic pain.

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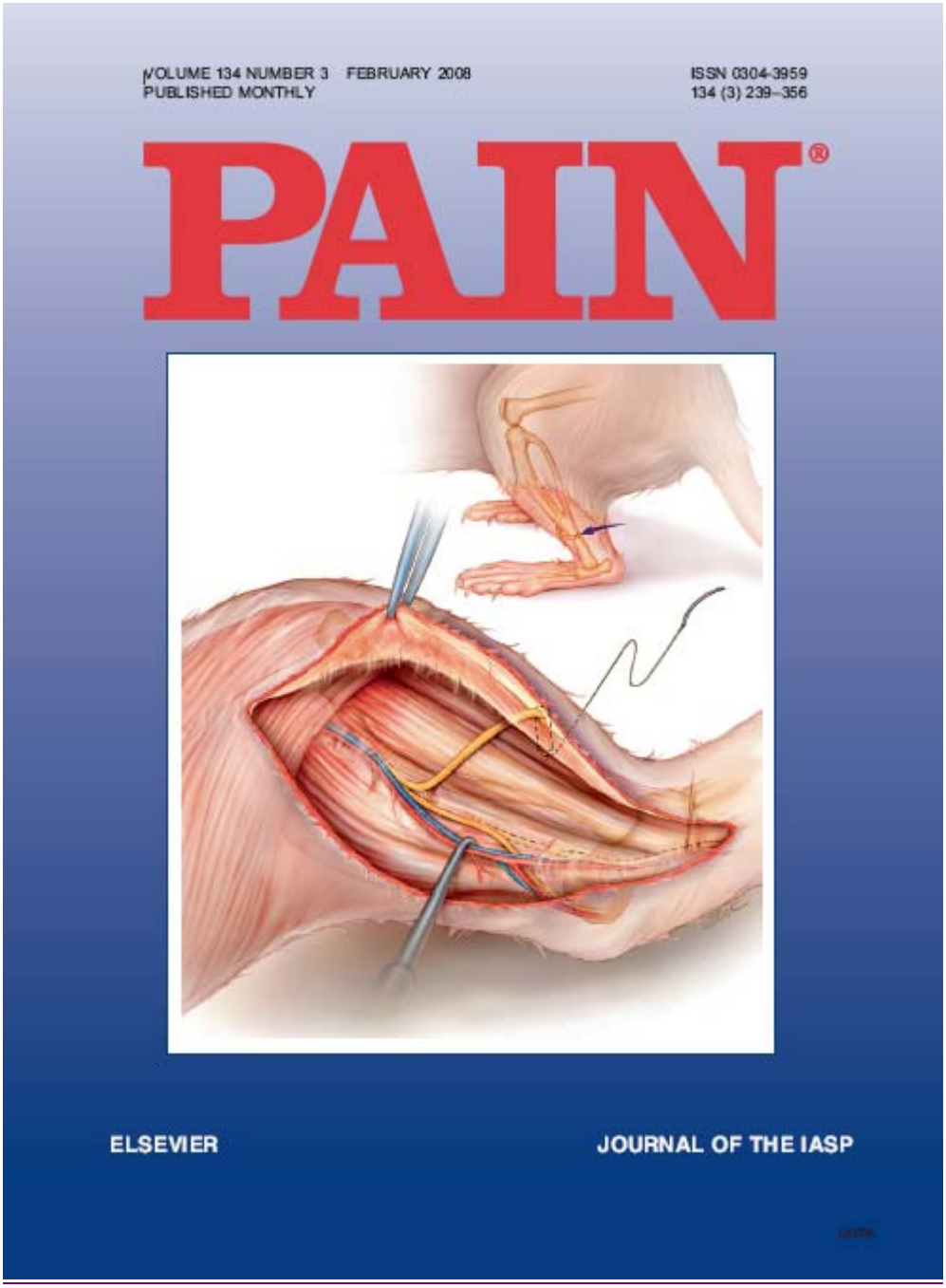
APPENDICES:



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The tibial neuroma transposition (TNT) model of neuroma pain and hyperalgesia

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Abstract

Peripheral nerve injury may lead to the formation of a painful neuroma. In patients, palpating the tissue overlying a neuroma evokes paraesthesias/dysaesthesias in the distribution of the injured nerve. Previous animal models of neuropathic pain have focused on the mechanical hyperalgesia and allodynia that develops at a location distant from the site of injury and not on the pain from direct stimulation of the neuroma. We describe a new animal model of neuroma pain in which the neuroma was located in a position that is accessible to mechanical testing and outside of the innervation territory of the injured nerve. This allowed testing of pain in response to mechanical stimulation of the neuroma (which we call neuroma tenderness) independent of pain due to mechanical hyperalgesia. In the tibial neuroma transposition (TNT) model, the posterior tibial nerve was ligated and transected in the foot just proximal to the plantar bifurcation. Using a subcutaneous tunnel, the end of the ligated nerve was positioned just superior to the lateral malleolus. Mechanical stimulation of the neuroma produced a profound withdrawal behavior that could be distinguished from the hyperalgesia that developed on the hind paw. The neuroma tenderness (but not the hyperalgesia) was reversed by local lidocaine injection and by proximal transection of the tibial nerve. Afferents originating from the neuroma exhibited spontaneous activity and responses to mechanical stimulation of the neuroma. The TNT model provides a useful tool to investigate the differential mechanisms underlying the neuroma tenderness and mechanical hyperalgesia associated with neuropathic pain.

Keywords: Neuroma; Neuropathic pain; Hyperalgesia; Nerve injury; Central sensitization; Allodynia; Neurofibroma

1. Introduction

Painful neuromas can arise from peripheral nerve injuries such as trauma, amputation, nerve biopsy, or resection of a neurofibroma. Patients experience tenderness to palpation of the skin overlying the neuroma, spontaneous burning pain, and allodynia and hyperalgesia in the distribution of the injured nerve. Despite advances in our understanding of neuropathic pain, providing adequate pain relief for these patients remains a clinical challenge. Unfortunately, a substantial proportion of patients develop pain that is refractory to contemporary pharmacological, psychological, and surgical intervention and endure significant disability. Therefore, research is needed to further increase our understanding of neuropathic pain and to develop novel therapies.

A number of animal models that involve traumatic nerve injuries have been developed to study neuropathic

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pain including sciatic nerve transection (Wall et al., 1979), chronic constriction injury (Bennett and Xie, 1988), partial sciatic nerve ligation (Seltzer et al., 1990), spinal nerve ligation (Kim and Chung, 1992), and spared nerve injury (Decosterd and Woolf, 2000). These contemporary models of neuropathic pain have greatly expanded our understanding of the mechanisms underlying neuropathic pain. However, several characteristics of these models limit their usefulness in studying neuroma pain. First, the observed behavioral changes are evoked by stimuli applied to the hindpaw at a location distant from the site of injury. There is currently no model measuring the effect of directly applying stimuli to the neuroma. Second, there is mounting evidence that hyperalgesia in the existing models can develop in the hindpaw independent of input from injured afferents and thus independent of the neuroma (Eschenfelder et al., 2000; Li et al., 2000). Further, hyperalgesia may develop following lesions that do not involve injury to afferent fibers (e.g., ventral rhizotomy) (Li et al., 2000; Sheth et al., 2002) or the formation of a neuroma (Eschenfelder et al., 2000; Sheth et al., 2002). These findings suggest that ectopic activity originating from a neuroma is not necessary for development of hyperalgesia.

We aimed to develop an animal model of neuroma pain. An ideal model would produce robust, severe, and lasting behavioral changes resembling those seen in patients with painful neuromas (i.e., ongoing pain sensations, pain evoked by palpation of the skin overlying the neuroma, and hyperalgesia in the distribution of the injured nerve). We propose that distinct but overlapping pathophysiological mechanisms underlie the multiple pain phenomena produced by peripheral nerve injury.

We based our model on the clinical observation that mechanical stimuli applied to the skin overlying a neuroma produce paraesthesias or lancinating pain in the distribution of the nerve (Hoffman-Tinel sign). It is believed that this clinical sign is indicative of ectopic mechanosensitivity of injured or regenerating afferent fibers. We hypothesize that mechanical stimuli applied to the skin overlying a neuroma in a rat will elicit a similar sensation and provoke foot withdrawal. Further, we hypothesize that mechanical hyperalgesia will develop in the cutaneous distribution of the injured peripheral nerve. Thus a peripheral nerve injury model was created that would permit the independent study of these two distinct pain behaviors.

2. Methods

2.1. Experimental animals

Eighty male Sprague–Dawley rats (Harlan, Indianapolis, IN) weighing 200–250 g were studied. Two to four animals were placed in plastic cages with sawdust bedding, housed in a climate controlled room under a 14/10 light/dark cycle, and provided food and water ad libitum. The animals were acclimatized under these conditions for at least a week before the initiation of experimentation. The Johns Hopkins University Animal Care and Use Committee approved the testing and surgical protocol.

2.2. Surgical procedures

The animals were randomly assigned to surgical groups for each experiment. For all surgical procedures, deep anesthesia was maintained throughout surgery with 2% isoflurane. All incisions were closed with running 6-0 silk sutures. All procedures were performed with a dissection microscope. At the conclusion of each experiment, all lesions were confirmed at autopsy.

2.2.1. Tibial neuroma transposition (TNT) model

The objective of the tibial neuroma transposition surgery was to produce a neuroma that was located in a position that was accessible for mechanical testing and that was outside of the innervation territory of the injured nerve. This allowed testing of pain in response to mechanical stimulation of the neuroma (which we call "neuroma tenderness") independent of pain due to hyperalgesia.

Our decision to use the tibial nerve was also based on the following factors: (1) The tibial nerve innervates the plantar surface of the hindlimb. The expected behavioral response to tibial neuroma stimulation would be hindlimb withdrawal. This behavior is easy to quantify and commonly used in most contemporary models of neuropathic pain. With experience we were able to increase the specificity of behavioral testing by scoring the intensity of hindpaw withdrawal. (2) The tibial nerve is a mixed nerve comprised of both sensory and motor nerve fibers. Thus, a tibial neuroma would be expected to develop electrophysiological properties similar to those demonstrated in other mixed nerve neuroma preparations (e.g. spinal nerves and sciatic nerve).

As illustrated in Fig. 1 (see also Fig. 3A), the posterior tibial nerve was exposed from approximately 8 mm proximal to the calcaneal branch to 1 mm distal to the plantar nerve bifurcation. The integrity of the calcaneal branch was preserved while it was dissected free from the main trunk of the tibial nerve. Just proximal to the plantar bifurcation, the tibial nerve was tightly ligated with 6-0 silk and sharply transected with scissors.

Using a blunt glass probe, a subcutaneous tunnel was burrowed from the medial incision site to the lateral aspect of the hindlimb. A 1.5 mm diameter plastic tube with a longitudinal slit in one wall was placed in the tunnel. The needle-bearing end of the suture used to ligate the tibial nerve was passed through the plastic tube and pushed through the skin at a location 8–10 mm superior to the lateral malleolus. The plastic tube was then removed from the tunnel. The suture was gently pulled to advance the tibial nerve stump through the subcutaneous tunnel, until it was flush with the inner surface of the skin of the lateral hind limb. The suture was then cut flush with the skin. The subsequent neuroma was located in a lateral position that was easily accessible for mechanical testing (Fig. 1, top). The suture material could be viewed just below the skin surface and provided a target for mechanical testing.

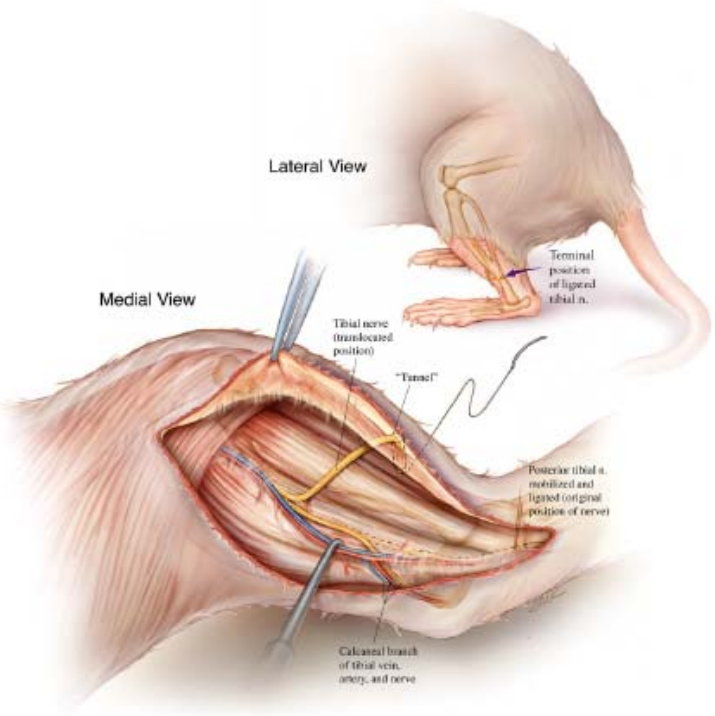


Fig. 1. The tibial neuroma transposition (TNT) model. Schematic depicting TNT model surgery. The distal tibial nerve in the foot is dissected free of adjacent tissue, ligated with a suture, and cut. The needle from the suture is passed through a subcutaneous tunnel to the lateral aspect of the hindlimb where it is pushed through the skin. The nerve is drawn into the tunnel until the ligature is adjacent to the skin. The suture is cut, and the incision closed (artwork by Ian Suk, Johns Hopkins University).

2.2.2. Sham surgery (S)

The tibial nerve was dissected as described above and left intact. A subcutaneous tunnel was formed as described above. A small piece of connective tissue was ligated and passed through the subcutaneous tunnel in the method described above (Fig. 3B).

2.2.3. Tibial neuroma with no transposition (TNT-nT)

The tibial nerve was dissected, ligated, and transected as described above for the TNT model surgery, but not transposed. A subcutaneous tunnel was formed and connective tissue was ligated and passed through to the lateral hindlimb as described above (Fig. 3C).

2.2.4. Tibial neuroma transposition with simultaneous proximal transection (TNT-sPT)

The TNT model surgery was performed as described above. Once the nerve stump was in place on the lateral aspect of the foot, the tibial nerve was sharply transected with scissors at the proximal entrance of the subcutaneous tunnel (Fig. 3D).

2.2.5. Tibial neuroma transposition with delayed proximal transection (TNT-dPT)

The TNT model surgery was performed as described above. Twelve days after surgery, the animals were re-anesthetized, and the tibial nerve was dissected free. Three millimeters proximal to the tunnel entrance, the nerve was tightly ligated with 6-0 silk and a 2–3 mm segment of the tibial nerve distal to the ligature was removed (Fig. 3E). A 6-0 silk suture was then used to anchor adjacent connective tissue to close the entrance to the tunnel. For control animals, the nerve was exposed but not cut.

2.3. Behavioral testing with mechanical stimuli

To insure blinding, the experimenters doing the behavioral testing were blinded to the surgery of each animal, and the different surgical groups were tested concurrently. The rats were tested three times preoperatively and several times during the postoperative period. The animals were placed in individual transparent plastic cages on top of an elevated wire mesh stage

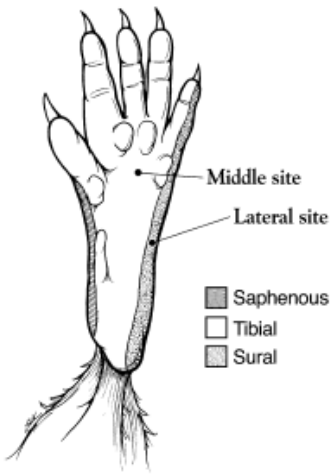


Fig. 2. Mechanical hyperalgesia testing sites. To test for mechanical hyperalgesia, von Frey probes were applied to lateral (sural distribution) or middle (tibial distribution) sites on the plantar surface of the paw. Nerve distributions were derived from Swett and Woolf (1985).

that allowed access to the plantar surface of the paw. A 2.5 × 20 cm window at the bottom of the sidewalls of the cages permitted application of von Frey filaments to the ankle region. The animals were allowed to acclimate to the testing environment for 20–30 min before testing began.

2.3.1. Neuroma tenderness

The suture tied to the distal end of the tibial nerve or connective tissue was visible through the skin and served as the target for mechanical stimuli. An analogous site served as the target on the contralateral hindlimb. A trial consisted of a train of five applications of a von Frey filament (150 mN for 1–2 s) with an interstimulus interval of 1 s. If the animal responded to any of the five applications, the trial was terminated. A positive response was defined as a slow withdrawal of the hindpaw, or rapid withdrawal with vocalization, licking, or shaking. Each testing session consisted of five trials to each hindlimb with an intertrial interval of about 2 min. The *Response Frequency* was defined as the percent of positive trials (i.e., 100 times the number of positive trials divided by five).

In later experiments, we implemented a grading system to qualitatively evaluate behavioral responses. Each trial was then assigned a response grade ranging from 0 to 2 based on the animal's response. A grade of 0 indicated that the animal did not respond during a given trial. A grade 1 response represented a slow withdrawal of the paw. A grade 2 response was defined as a brisk withdrawal or shaking, licking, or vocalization. The *Withdrawal Score* was defined as the sum of response grades for the five trials and ranged from 0 to 10.

Whether the response was specific to mechanical stimuli applied to the target site was evaluated in a small cohort of animals following TNT surgery. Testing was performed as described above, but in addition to the neuroma test site, stimuli were applied to skin overlying the tibial nerve 3 mm proximal to the neuroma, and the skin of the lateral hindlimb 3 mm and 5 mm inferior to the neuroma.

2.3.2. Hindpaw mechanical hyperalgesia

Mechanical withdrawal threshold to the application of a von Frey probe to the foot was measured by using the up-down method (Dixon, 1980). An ascending series of von Frey hairs of logarithmically incremental force (3.2, 5.2, 8.3, 15, 29, 44, 64, 94, and 160 mN) were applied to sites in the middle (tibial nerve distribution) and lateral (sural nerve distribution) aspect of the plantar surface of the left hindpaw (Fig. 2). Mechanical testing followed the procedure described by Ringkamp et al. (1999). Each von Frey hair was applied to the test area for about 2–3 s, with a 1–2 min interval between stimuli. A trial began with the application of the 15 mN von Frey probe to the left and right hindpaws of each animal. A positive response was defined as a rapid withdrawal and/or licking of the paw immediately upon application of the stimulus. Whenever a positive response to a stimulus occurred, the next smaller von Frey hair was applied, and whenever a negative response occurred, the next higher force was applied. The testing continued for five more stimuli after the first change in response occurred, and the pattern of responses was converted to a 50% von Frey threshold using the technique described by Dixon (1980). If the animal showed no response to the highest von Frey hair (160 mN), a von Frey threshold of 260 mN, corresponding to the next log increment in potential von Frey probes, was assigned to the threshold.

2.4. Lidocaine block

Nine weeks following TNT surgery, a cohort of eight rats displaying elevated behavioral response frequencies to mechanical stimulation of the neuroma and plantar mechanical hyperalgesia were randomly assigned to two interventional groups, local lidocaine injection or control lidocaine injection. In pairs, the animals were lightly anesthetized using 2% isoflurane. One animal received a 100 μl injection of 1% lidocaine with epinephrine to the neuroma target site marked by the suture on the tibial nerve. To control for systemic effects of lidocaine, the lidocaine/epinephrine was injected into the subcutaneous tissue overlying the lumbar spine of the other animal. Ten minutes after awakening from anesthesia, the animals were placed in cages on top of the testing stage as described above. Blinded behavioral testing of the neuroma and hindpaw was performed as described above immediately prior to lidocaine injection and three times after injection (15 min, 60 min, 120 min). Two days later, the animals were crossed over to the other treatment arm and the behavioral protocol was repeated.

2.5. Electrophysiological procedures

The rats were initially anesthetized with pentobarbital (50 mg/kg, intraperitoneal). Anesthesia was maintained by intravenous administration of pentobarbital (8–10 mg/kg/h) via the jugular vein. Heart rate was continuously monitored as an indicator of adequate anesthesia. A tracheotomy was

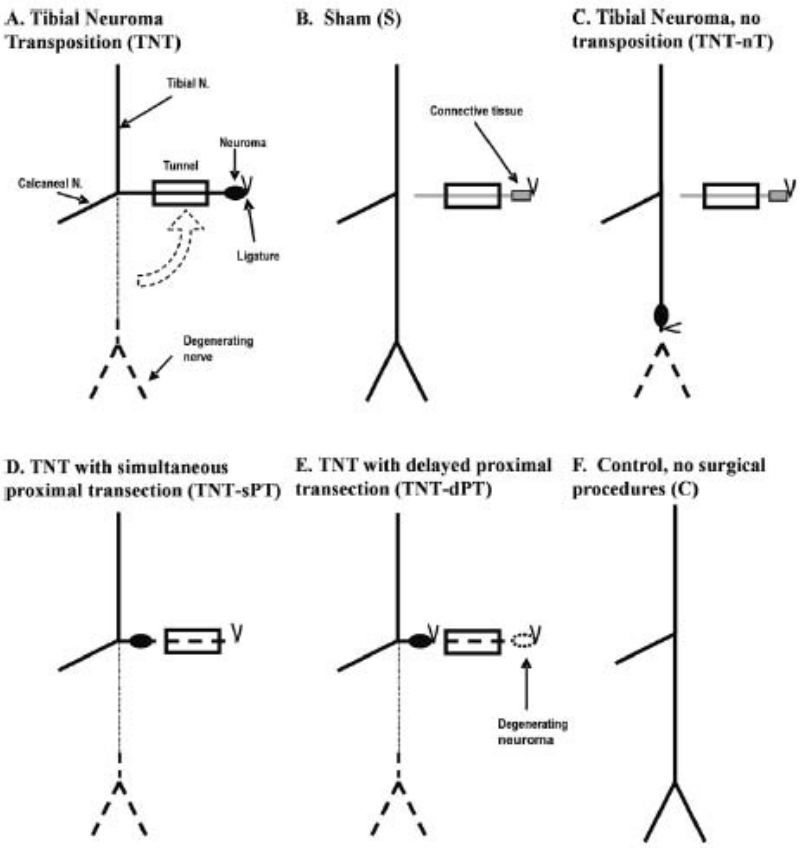


Fig. 3. Schematic of the different surgical groups.

performed, and animals were artificially ventilated to maintain expired pCO_2 to 40 mm Hg. Muscle paralysis was achieved by intravenous pancuronium bromide (1 mg/kg). Feedback-controlled, water-perfused heating pads were used to maintain core temperature (measured by a rectal probe) at 38 °C.

Electrophysiological recordings were made from the tibial nerve. Teased-fiber recording techniques were used as described previously (Campbell and Meyer, 1983). Briefly, a skin incision was made above the tibial nerve in the popliteal fossa, and the tibial nerve was exposed. The skin around the incision was used to form a pool by suturing the edges to a metal ring. The pool was filled with warm paraffin oil. A splitting platform was placed underneath the nerve at the proximal end, and a small silver wire which served as the recording electrode was positioned above the splitting platform. Small bundles were cut from the nerve, and teased into small filaments suitable for recording activity from single fibers. A stimulating electrode was placed under the nerve at the distal end of the incision, about 1.5 cm distal to the recording electrode. The stimulation electrode was used to deliver electrical pulses of variable strength to the nerve in order to count the number of A and C fibers on the recording electrode.

The neural signal was differentially amplified, filtered, and digitized at a rate of 25 kHz. A real-time computer-based data acquisition and processing system (DAPSYS, Brian Turnquist, Johns Hopkins University; for details, go to http://www.dapsys.net) was used to record neural activity. The software provided multiple window discriminators for real-time sorting of different action potential waveforms. All waveforms passing a selectable threshold level were saved for post hoc analysis.

Neural recordings were performed 100–120 days after the neuroma (or sham) surgery. After determining the number of fibers at the recording electrode that responded to electrical stimulation of the nerve, spontaneous activity was measured over a 5 min interval. A heat lamp was then applied to determine if the spontaneous activity originated from cold or warm

fibers. The skin over the neuroma site was stimulated with a von Frey probe (150 mN) and blunt pressure to determine whether mechanically sensitive fibers were present.

2.6. Histological procedures

Animals were euthanized by cardiac puncture under deep anesthesia and subsequently perfused with saline and 4% paraformaldehyde in Sorenson's buffered solution. Sections from the tibial nerve proximal and distal to all sites of ligation and transection, as well as the neuroma were harvested. The specimens were post-fixed in 2% osmium tetroxide and embedded in Epon. Sections (1 μm) were stained with toluidine blue.

2.7. Experimental design

Three separate groups of animals were included in the experiments described in this study.

2.7.1. Experiment group one

The aim of the initial experiment group was to demonstrate that the TNT model surgery led to the formation of a neuroma with characteristic electrophysiological and histological properties and also led to the development of a behavioral response that could be evoked by applying mechanical stimuli to the skin overlying the neuroma. The TNT surgery and the three different control procedures performed in experiment group one are illustrated in Fig. 3. The TNT model surgery was performed in eight animals (Fig. 3A). The three different control procedures were aimed at confirming that the pain behavior in response to palpating the ligature site was due to the neuroma formation. These control procedures were performed concurrently and were therefore also useful in blinding the experimenters. For eight animals, the TNT model surgery was performed, but the tibial nerve was simultaneously transected proximal to the tunnel (TNT-sPT, Fig. 3D). For eight additional animals, the tibial nerve was ligated and cut but not transposed to the lateral location (TNT-nT, Fig. 3C). Finally, the tibial nerve was exposed but not cut in eight sham animals (S, Fig. 3B). For all animals, a tunnel was created and a suture was placed under the skin. Behavioral testing for mechanical sensitivity at the neuroma test site was performed in all of the animals. In animals with the TNT, mapping of the behavioral response following application of stimuli at sites distant from the neuroma was also performed. Electrophysiological and histological studies were performed on a subset of these animals at the conclusion of the behavioral studies.

2.7.2. Experiment group two

The aim of the second experimental group was to investigate the effects of the TNT model on paw withdrawal thresholds to mechanical stimuli applied to the plantar surface of the hindpaw. In addition to TNT surgery ($N = 8$), two control groups were included in this experiment group. Eight animals underwent a sham procedure ("S", Fig. 3B), and eight animals did not undergo any surgical procedures ("C", Fig. 3F). All animals were tested for mechanical hyperalgesia in the hindpaw, as well as mechanical sensitivity at the lateral ankle (i.e., the neuroma test site). At the conclusion of eight weeks of behavioral testing, the eight animals that had received the TNT model surgery were selected for the lidocaine experimental protocol described above.

2.7.3. Experiment group three

The aim of the third experiment group was to determine if the behaviors provoked by applying mechanical stimuli to neuroma test site or plantar hindpaw depended on the presence of the neuroma at the lateral testing site. Twenty-four animals underwent TNT model surgery. Twenty animals that demonstrated robust neuroma tenderness and plantar hyperalgesia were selected and divided into two surgical groups. Ten days after the TNT surgery, 11 animals received a delayed proximal transection of the tibial nerve (TNT-dPT, Fig. 3E). To control the effects of re-exposing the tibial nerve, the tibial nerve was exposed but left intact in the remaining nine animals. All animals underwent additional behavioral testing of the neuroma test site and lateral aspect of the plantar hindpaw.

2.8. Statistical analysis

Since the behavioral scoring methods employed yield discrete prefixed values rather than a continuum, and since the data were not normally distributed because of the ceiling effects of a limited range of von Frey hairs, non-parametric tests were performed. Tests were performed to analyze the variance between testing days (Friedman ANOVA for repeated measurements, followed by Wilcoxon matched pairs when appropriate) and between surgical groups on a given testing day (Kruskal–Wallis ANOVA, followed by Mann–Whitney U-test when appropriate). A p value of <0.05 was considered to be statistically significant. Data are presented as median with 25th and 75th quartiles.

3. Results

3.1. Mechanical stimulation of the skin overlying a tibial neuroma produces a behavioral response

In experiment one, eight animals underwent the tibial neuroma transposition (TNT) surgery in which the tibial nerve was ligated and rotated such that the ligature was positioned at the lateral side of the ankle (Fig. 3A). The three control groups in experiment one are illustrated in Figs. 3B–D. Mechanical stimulation of the lateral side of the ankle with a von Frey probe normally did not lead to a behavioral response. However, following the TNT surgery, animals developed a vigorous response to von Frey stimulation at the ligature (which could be visualized through the skin). The incidence of response to five trials of mechanical stimulation is plotted as a function of time after the lesion in Fig. 4. The response frequency for the TNT group differed significantly from baseline starting on postoperative day 5 and persisting for the duration of the experiment (100 days). The response frequency for the TNT group differed from the three control groups starting on day six and for most of the time points thereafter.

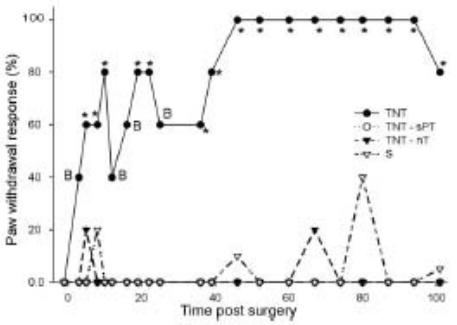


Fig. 4. TNT model produces neuroma tenderness. Following TNT surgery, animals displayed an increased frequency of response to application of a 150 mN von Frey probe to the ligature site. The median behavioral response frequency for the TNT group differed significantly from baseline starting on postoperative day 5 ($B = p < 0.05$). The TNT group differed significantly from the three control groups starting on day 7 ($^{*} = p < 0.05$ with respect to baseline and with respect to other groups). The control groups did not differ significantly from baseline or each other. Schematics of the surgeries performed in each of the groups are shown in Fig. 3.

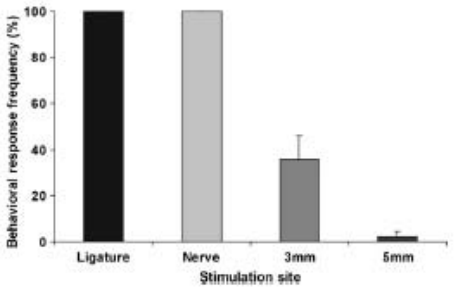


Fig. 5. Focal region of neuroma tenderness in TNT model. The behavioral response frequency to application of a 150 mN von Frey probe was measured at four sites on the lateral hindlimb: the ligature site, 3 mm proximal to the ligature (on the tibial nerve), 3 mm inferior to the ligature, and 5 mm inferior to the ligature ($n = 9$).

There was no consistent difference in response frequency for any of the control groups compared to baseline or each other. These control groups were run concurrently with the TNT model animals to insure blinding of the experimenter. Perhaps the most interesting control group is the TNT-sPT group in which the tibial nerve was ligated and rotated as is done for the TNT model surgery but the tibial nerve was cut simultaneously about 1 cm proximal to the ligature. This group did not display an increased response to mechanical stimulation at the ligature site indicating that the behavior was not due to the surgical manipulations necessary to position the ligature on the lateral side of the foot, but rather require that the nervous supply to the ligature site (and eventual neuroma) was intact.

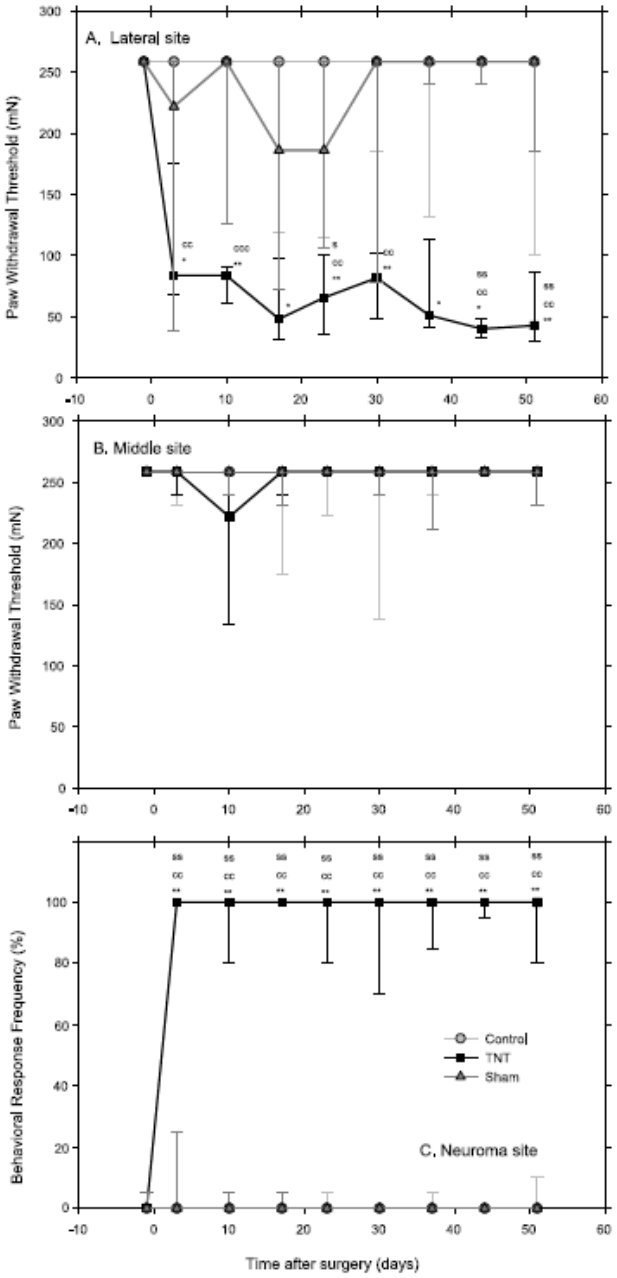
To confirm that this behavior did not reflect cutaneous hyperalgesia but rather required stimulation of the neuroma, we applied the von Frey probe at four different locations relative to the ligature (Fig. 5). Von Frey stimulation to the skin overlying the ligature or along the course of the tibial nerve 3 mm proximal to the ligature always evoked a 100% response in all animals. Response frequencies decreased in a distance dependent manner as the probe was applied 3 mm ($RF = 36 \pm 10\%$) and 5 mm ($RF = 2 \pm 2\%$) inferior to the ligature test site.

3.2. The TNT surgery produces behavioral signs of mechanical hyperalgesia in the hindpaw

Experiment two served as a randomized, controlled assay for the development of mechanical hyperalgesia in the hindpaw following TNT model surgery. Paw withdrawal thresholds to mechanical stimuli applied to the lateral paw and middle paw are shown in Fig. 6. At baseline, there was no difference in withdrawal thresholds at either site amongst the groups. In all groups, the withdrawal thresholds in the middle of the hindpaw (tibial nerve distribution) were at or near the cut off value for all time points, reflecting the fact that the animals did not respond to the highest von Frey before the injury and that the middle of the paw is almost completely denervated by the tibial lesion.

The mechanical withdrawal thresholds in the lateral aspect of the hindpaw varied significantly with group and time. Animals in the TNT model group displayed mechanical withdrawal thresholds that were significantly lower than baseline and the naive control group for the duration of the postoperative period (51 days) with the exception of days 17 and 37 when they were only significantly lower than baseline. The TNT model group displayed paw withdrawal thresholds that tended to be lower than those of the sham group on all postop-

Fig. 6. TNT model produces mechanical hyperalgesia. Paw withdrawal thresholds to von Frey stimuli applied to the lateral (A) and middle (B) test sites are plotted as a function of time after the surgery. (A) At the lateral test site, animals in the TNT model group (filled square, $n = 8$) displayed mechanical withdrawal thresholds that were significantly lower than baseline ($^{*} = p \leqslant 0.05$, $^{**} = p \leqslant 0.01$), the non-operated control (circle, $n = 8$, $^{\circ} = p \leqslant 0.05$, $^{\circ\circ} = p \leqslant 0.01$), and the sham group (triangle, $n = 8$, $^{\#} = p \leqslant 0.05$, $^{\#\#} = p \leqslant 0.01$). There was no difference in withdrawal thresholds at either site amongst the groups at baseline. (B) In all groups, the withdrawal thresholds in the middle of the hindpaw (tibial nerve distribution) did not vary significantly from baseline at any point in the postoperative period. (C) Tenderness over the lateral ankle developed in all animals following TNT model surgery, but not in the sham or control animals. The response frequencies for the TNT model group were significantly elevated compared to non-operated control group ($^{\circ\circ} = p \leqslant 0.01$), the sham group ($^{\#\#} = p \leqslant 0.01$), and baseline ($^{**} = p \leqslant 0.01$).


A, Lateral site
B, Middle site
C, Neuroma site
Paw Withdrawal Threshold (mN)
Behavioral Response Frequency (%)
Time after surgery (days)
Control
TNT
Sham

erative days. This difference reached significance on days 23, 44, and 51. For the sham and naive groups, lateral-site paw withdrawal thresholds did not vary significantly from baseline or each other.

3.3. Proximal tibial nerve transection reverses the neuroma tenderness produced by the TNT model

Experiment three evaluated the effect of a delayed, proximal tibial nerve transection on neuroma tenderness. Twenty of the twenty-four animals that underwent TNT model surgery displayed behavioral response scores that were significantly greater than baseline six days after surgery. Ten days after the TNT model surgery, 11 of these animals underwent proximal tibial nerve transection. The remaining 9 animals had the tibial nerve exposed (but not cut) and served as the control animals to blind the experimenter.

Following proximal tibial nerve transection, behavioral response scores for stimulation at the neuroma dropped abruptly and were not significantly different from the baseline scores before the TNT surgery. The scores were significantly lower than immediately prior to proximal tibial nerve transection for the entire testing period (Fig. 7). In contrast, following tibial nerve exposure in the control group, response scores did not decrease but remained significantly greater than baseline and did not vary significantly from immediately prior to tibial nerve exposure. Behavioral response scores were significantly lower for the proximal tibial nerve transection compared to tibial nerve exposure group on all postoperative test days. Thus, proximal tibial nerve transection led to a reversal of the neuroma pain behavior.

3.4. Mechanical hyperalgesia in the hindpaw produced by the TNT model persists following proximal tibial nerve transection

Experiment three was also used to assess the effects of proximal tibial nerve transection on mechanical hyperalgesia produced by TNT model surgery. At baseline, paw withdrawal thresholds for the two groups did not differ (Fig. 7B). Immediately after TNT model surgery, paw withdrawal thresholds on the lateral side of the foot were significantly lower than baseline for both groups. No difference was evident between the groups. Following proximal tibial nerve exposure or transection, paw withdrawal thresholds remained significantly decreased from baseline for all animals. There was no difference in paw withdrawal threshold between the two groups at any time point. Thus, proximal tibial nerve transection did not lead to a reversal of the hindpaw hyperalgesia.

A total of 40 animals received TNT surgery in the three experimental groups. Thirty-six of these animals (90%) displayed a positive behavioral response to mechanical stimulation of the skin overlying the neuroma.

3.5. Local lidocaine injection reverses the neuroma tenderness produced by the TNT model, but does not effect hindpaw mechanical hyperalgesia

Fig. 8 illustrates the effects of local lidocaine injection on neuroma tenderness and hindpaw mechanical hyperalgesia compared to the effects of lidocaine injection at a remote site. Eight TNT animals from experiment group two that displayed increased behavioral response frequencies at the neuroma-site and hindpaw mechanical hyperalgesia nine weeks after tibial nerve neuroma model surgery were enrolled in a crossover study. Response frequencies were significantly lowered following local, but not remote, lidocaine injection. This was first evident at 15 min and lasted for the duration of the experiment (120 min). Behavioral response frequencies following remote lidocaine injection did not significantly differ from pre-injection levels.

Paw withdrawal thresholds to mechanical stimuli applied to the lateral aspect of the paw did not differ from pre-injection levels following remote or local lidocaine injection. Following injection, the withdrawal thresholds of the two groups did not differ with respect to each other.

3.6. TNT model surgery results in the formation of a histologically characteristic neuroma

Histological sections of the neuroma, proximal nerve and distal nerve stump were examined at the completion of the experiments, some seven months after creation of the neuroma. Longitudinal sections through the neuroma demonstrated demyelination, enlarged unmyelinated axons, increased collagen, excess endoneurial cells and chaotic orientation of axons, all features characteristic of nerve-end neuromas that do not undergo rotation (Fried and Devor, 1988). In the area of the neuroma nearest the ligature, there were numerous, large, unmyelinated axons as previously observed in detailed studies of neuroma endbulb formation (Fried et al., 1991). Unlike previous studies, we observed a significant number of thinly myelinated axons within several hundred microns of the ligature site (Fig. 9), presumably reflecting very late changes in these neuromas studied more than 200 days after nerve ligation. More proximal parts of the nerve, leading to the neuroma, exhibited a normal density of axons and the formation of some regenerative clusters.

The distal nerve stumps that were generated at the time of the initial injury were identified at autopsy by microdissection. Perhaps surprisingly, these distal nerve stumps were not denervated but rather exhibited a large number of axons, although the number was markedly reduced when compared to uninjured nerves. The origin

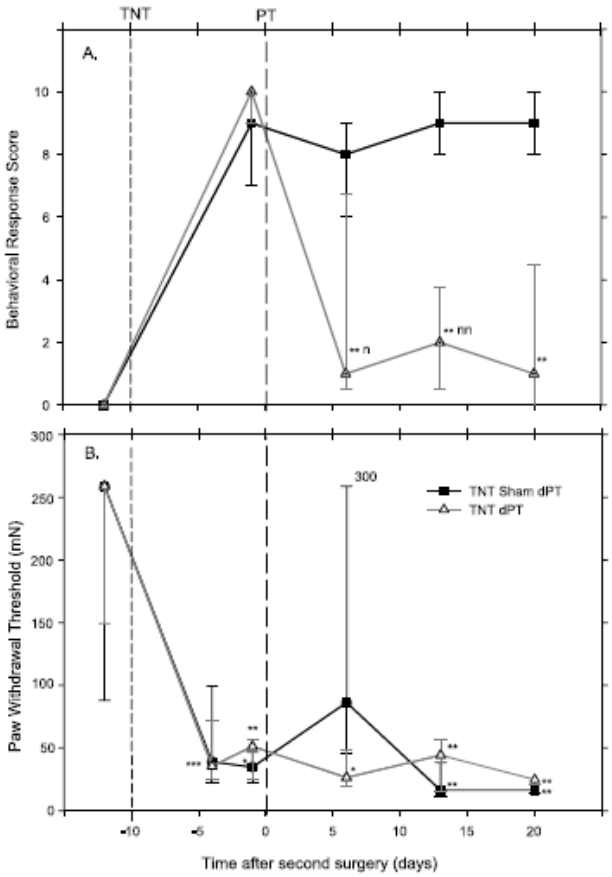


Fig. 7. Proximal tibial nerve transection reverses neuroma tenderness. The TNT model surgery was done on all animals. Ten days later, the tibial nerve was exposed 1 cm proximal to the ligature site in animals that had developed robust pain behaviors ($n = 20$). The nerve was transected in 11 of the animals (TNT-dPT) and left alone in the others (TNT sham dPT). (A) Neuroma tenderness is reversed by proximal tibial nerve transection. The TNT surgery produced behavioral response scores that were significantly greater than baseline for both groups. The delayed proximal transection (dPT), but not sham, resulted in a significant decrease in the behavioral response scores to a level that was not significantly different from baseline. The behavioral response scores remained significantly lower than immediately following TNT model ($^{**} = p \leqslant 0.01$) for the entire testing period. Compared to sham dPT group, the dPT group demonstrated behavioral response scores that were significantly lower on all postoperative test days ($^{n} = p \leqslant 0.05$, $^{nn} = p \leqslant 0.01$). (B) Paw hyperalgesia is not changed by proximal tibial nerve transection. At baseline, paw withdrawal thresholds for the two groups did not differ. Immediately after the TNT surgery, paw withdrawal thresholds were significantly lower than baseline for both groups. No difference was evident between the groups. Following dPT or sham dPT, paw withdrawal thresholds remained significantly decreased from baseline and did not differ from post-TNT model levels ($^{*} = p \leqslant 0.05$, $^{**} = p \leqslant 0.01$, $^{***} = p \leqslant 0.001$). There was no difference in paw withdrawal threshold between the two groups at any time point.

and directionality of these axons was not established in these experiments, and it is possible that these axons were retrograde or anterograde in direction (Belzberg and Campbell, 1998). As described below, we demonstrate that these axons did not arise from the tibial nerve itself.

Proximal tibial nerve transection (Experiment three) resulted in massive Wallerian degeneration in the nerve-end neuromas when examined on day 5 post-transection. Following proximal tibial nerve transection, however, there was no Wallerian degeneration seen in the distal nerve stump indicating that repopulation of

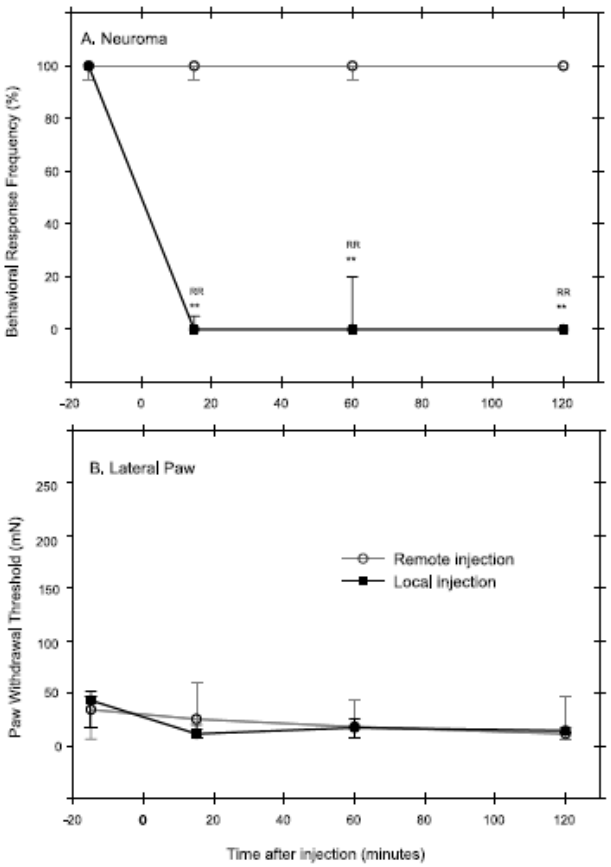


Fig. 8. Lidocaine injection at the site of the neuroma reverses neuroma tenderness. Nine weeks after TNT surgery, lidocaine (1%, 100 μl) was injected at the site of the neuroma or at a remote site. (A) The local lidocaine injection resulted in a significant decrease in the response frequency to mechanical stimulation of the neuroma (** $= p \leqslant 0.01$). Injection of lidocaine to a remote site did not affect the response frequency. The response frequencies were significantly lower following injection for the local versus remote injection group for the entire 120-min test period ($^{RR} = p \leqslant 0.01$). (B) Lidocaine injection, local or remote, did not alter paw withdrawal thresholds to mechanical stimulation of the plantar hindpaw at any time point following injection.

the distal nerve stump was not due to invasion by axons arising from the tibial nerve but rather to recruitment of axons from other nerves. This suggests that reinnervation of the plantar skin may be due in part to axons from adjacent nerves that have regenerated through the distal stump of the tibial nerve.

3.7. Afferent fibers originating from the tibial neuroma exhibited spontaneous activity and mechanosensitivity

In animals with a tibial nerve neuroma ($n = 3$), single fiber recordings were obtained from 130 units (90 C fibers and 40 A fibers) in the tibial nerve. Spontaneous activity was observed in 14 fibers; in two of these fibers the spontaneous activity originated from cold fibers since it was stopped by gentle warming. Mechanical stimulation of the neuroma elicited a response in 5 fibers. In the sham animal, single fiber recordings were obtained from 26 fibers (19 C fibers and 7 A fibers). Spontaneous activity was observed in 2 fibers; both were cold fibers since gentle warming stopped the spontaneous activity. Mechanical stimulation over the nerve did not elicit a response. These results are comparable to those obtained by others who have recorded from neu-

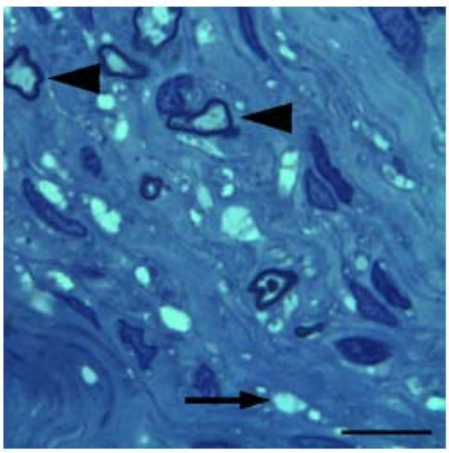


Fig. 9. Thinly myelinated axons in long-term neuromas. Longitudinal sections through a representative neuroma at seven months after surgery demonstrate thinly myelinated axons in both longitudinal and transverse orientation (arrowheads). Large unmyelinated axons, typical of endbulbs, are also seen (arrow). There is an increase in unmyelinated axons, non-neuronal endoneurial cells and collagen. Bar equals 40 μ.

romas in the peripheral nerve (Blumberg and Jänig, 1984; Meyer et al., 1985).

4. Discussion

We developed a novel model of neuropathic pain to specifically investigate the mechanisms of neuroma pain. The tibial neuroma transposition (TNT) model produces a robust and lasting behavioral response characterized by tenderness to mechanical stimuli applied over the neuroma and hyperalgesia to mechanical stimuli applied to the plantar hindpaw. The neural mechanisms of neuroma tenderness and mechanical hyperalgesia appear to be different since interruption of the pain signaling pathway from the neuroma to the CNS by local application of lidocaine or by proximal transection of the tibial nerve eliminates the neuroma tenderness but not the plantar mechanical hyperalgesia.

The basis for the TNT model is the clinical observation that palpating the tissue overlying a neuroma evokes paraesthesias/dysaesthesias in the distribution of the injured nerve. Anecdotal reports indicate that palpating sciatic nerve neuromas in rats evokes distress vocalization and struggling (Devor et al., 1999). The TNT model is the first animal model that allows neuroma tenderness to be systematically evaluated.

4.1. Neuroma tenderness

Several measures were taken to ensure that the behavioral response was dependent on the stimulation of the neuroma. To avoid the possibility that the behavioral response was due to the hyperalgesia often seen in the distribution of an injured nerve or due to incisional pain, the tibial neuroma was rotated from its natural position to the lateral aspect of the hindlimb. Control groups for the different surgical steps in the TNT model were used to exclude behaviors associated with surgically damaged soft tissue. Maps of the area of mechanical hypersensitivity demonstrated that the evoked behavior was specific to stimuli applied to the neuroma and not to hyperalgesia of the adjacent tissue. Finally, we were able to reverse the neuroma tenderness by interrupting signaling from the neuroma to the CNS.

The severity, robustness, and duration of neuropathic pain behaviors produced by the TNT model are comparable to those of other models of neuropathic pain including the chronic constriction injury (Bennett and Xie, 1988), partial sciatic nerve ligation (Seltzer et al., 1990), spinal nerve ligation (Kim and Chung, 1992), and a spared nerve injury (Decosterd and Woolf, 2000). The neuroma tenderness appeared within several days.

Within hours of a nerve transection, ectopic mechanosensitivity develops at the severed nerve tips (Welk et al., 1990; Koschorke et al., 1991; Michaelis et al., 1995). In myelinated fibers, the incidence of mechanically sensitivity increases over the first 24 h reaching a level of about 25%. This is presumably due to the axonal transport and accumulation of transduction elements at the severed tip (Koschorke et al., 1994). For unmyelinated fibers, the incidence of mechanosensitivity is about 13% and remains relatively constant over a 2-month period (Welk et al., 1990). Afferent fibers whose regenerating sprouts become trapped in neuromas also develop ectopic spontaneous activity, crosstalk, and sensitivity to thermal and chemical stimuli (Blumberg and Jänig, 1984; Devor et al., 1999; Michaelis et al., 1999; Rivera et al., 2000). Our findings of spontaneous activity and mechanically-evoked responses in A-fiber and C-fiber afferents in the tibial neuromas are consistent with these reports.

The ectopic mechanosensitivity of afferents trapped in the neuroma is believed to be responsible for the abnormal sensory phenomena evoked by neuroma palpation. Microneurography in a patient with a peroneal nerve neuroma revealed that percussion of the neuroma elicited an intense burst of spike activity and augmentation of the patient's pain (Nyström and Hagbarth, 1981). In experimental neuroma preparations, 'hot spots' of mechanosensitivity are clustered at the nerve endbulb (Devor et al., 1999). Following a crush injury or nerve section with resuturing, mechanosensitive sites

have been observed up to 6 mm proximal to the injury site (Gorodetskaya et al., 2003) presumably due to retrograde sprouting. Our finding that mechanical stimulation of the tibial nerve trunk proximal to the neuroma evoked a behavioral response is consistent with the existence of mechanosensitive spots proximal to the injury site.

Changes in the phenotype, quantity, and distribution of ion channels (specifically sodium channels that accumulate in the nerve stump) may underlie the ectopic electrical properties that arise in injured afferents (Devor et al., 1999). Systemic or topical application of a range of "membrane stabilizers" (Na channel blockers) rapidly silences abnormal firing generated at nerve-injury sites in rats (Yaari and Devor, 1985; Burchiel, 1988; Devor et al., 1994; Matzner and Devor, 1994). Further, perioneuromal or trigger point injections of local anesthetic often provide relief for patients with painful neuromas (Chabal et al., 1992; Gracely et al., 1992). Consistent with this latter observation is our finding that the neuroma tenderness is reversed by local lidocaine injection.

4.2. Plantar hyperalgesia

Following nerve injury, patients report allodynia and hyperalgesia in the partly denervated skin (Trotter and Davies, 1909; Sunderland, 1978; Fishbain et al., 1996; Bonica et al., 2001). An advantage of the TNT model is that it produces both mechanical hyperalgesia in the cutaneous territory of the sural nerve and neuroma tenderness. The sural nerve territory lies adjacent to and partially overlaps the denervated tibial nerve territory (Swett and Woolf, 1985). Similarly, lesion of two of three terminal branches of the sciatic nerve (tibial and peroneal) also produces robust mechanical hyperalgesia in the cutaneous territories of the spared sural and saphenous nerves (Decosterd and Woolf, 2000).

In the present study, proximal tibial nerve transection reversed neuroma tenderness, but not plantar mechanical hyperalgesia. This supports the hypothesis that the two behaviors have distinct mechanisms. The neuroma tenderness is dependent on activity originating from the neuroma. The persistence of plantar mechanical hyperalgesia suggests that this hyperalgesia is independent of ectopic activity from the neuroma.

The plantar hyperalgesia seen in this model is similar to that seen in other neuropathic models involving traumatic nerve injuries and is likely due to similar mechanisms. Many authors think that hyperalgesia is a result of central sensitization to input from normal afferents. What drives this central sensitization is controversial. Ectopic activity from injured afferents appears to play a role in some studies (Liu et al., 2000). Another possibility is that adjacent, uninjured nociceptive afferents develop spontaneous activity (Wu et al., 2001) that might drive central sensitization. A recent study reported that uninjured nociceptors become sensitized to mechanical stimuli after a spinal nerve ligation injury (Shim et al., 2005), and therefore central sensitization may not be required.

Since behavioral testing was not performed until several days after the proximal tibial nerve transection, we cannot exclude the possibility that a new neuroma developed at the transection site and became the focus of ectopic impulse generation that could drive the central sensitization responsible for the plantar hyperalgesia. For example, spontaneous activity in unmyelinated afferents can develop within 30 h of nerve section (Michaelis et al., 1995). However, lidocaine injections at the neuroma site reversed the neuroma tenderness (and presumably ectopic activity from the injury site) but also did not reverse the hyperalgesia. These manipulations did not block ectopic impulses in injured afferents that may arise from more proximal locations along the nerve trunk or the DRG.

4.3. Ongoing pain

Patients describe ongoing burning, cramping, or lancinating sensations in the distribution of the injured nerve. Ongoing pain may be due, at least in part, to movement of the neuroma which is tethered to adjacent tissue since surgical repositioning of the neuroma to minimize movement can alleviate some of the pain. Measurements of spontaneous pain in animals have been problematic. Several authors have advocated that self-mutilating behavior, termed autotomy, observed after sciatic nerve transection is an indication of spontaneous pain (Wall et al., 1979; Levitt, 1985; Coderre et al., 1986; Blumenkopf and Lipman, 1991; Seltzer et al., 1991). Others argue that the autotomy behavior represents a reaction to chronic paraesthesias, excessive grooming, or a proclivity of some species to shed a functionally impaired insensate limb (Rodin and Kruger, 1984; Lindblad and Ekenvall, 1986; Moossy et al., 1987). Although the TNT model produces ectopic electrical activity and stimulus evoked pain behaviors, none of the animals exhibited autotomy. Other forms of spontaneous pain behaviors (e.g., hindlimb flinching, scratching, or biting) were not observed. Therefore, the presence and/or degree of spontaneous pain produced by the TNT model remains uncertain.

4.4. Clinical relevance

Many of the drugs currently used to treat neuropathic pain result in unacceptable side effects such as sedation and cognition impairment. The TNT model will be an important tool in the preclinical development of new therapies for neuropathic pain. The TNT model allows neuroma tenderness to be investigated indepen-

dent of hyperalgesia. This provides the opportunity to investigate novel therapeutic strategies that specifically target neuroma pain.

Acknowledgments

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Supporting Data

Has been placed within the body of the report